

Targeting the environmental assessment of veterinary drugs with the multi-species-soil system (MS·3) agricultural soil microcosms: the ivermectin case study

G. Carbonell-Martin*, J. Pro-Gonzalez, P. Aragonese-Grunert, M. M. Babin-Vich, C. Fernandez-Torija and J. V. Tarazona-Lafarga

Laboratory for Ecotoxicology. Department of the Environment. INIA. Ctra. A Coruña, km 7,5. 28040 Madrid. Spain

Abstract

The environmental risk assessment of the veterinary pharmaceutical ivermectin is receiving significant attention. This paper assesses the capacity of the MS·3 soil microcosm as a tool for targeting the environmental impact assessment of veterinary drugs, using ivermectin as model. Two screening MS·3 were performed using different European soils; one with a soil collected in an agricultural station near to Madrid, Spain and a second with a soil collected in a farm area close to York, UK. Soils were fortified with ivermectin at the following ranges: 0.01-10 mg kg⁻¹ and 0.1-100 mg kg⁻¹ in the Madrid and York studies, respectively. The effects on earthworms, plants and soil microorganisms were assessed in the Madrid soil. Toxicity tests on aquatic organisms (algae, cladocerans and *in vitro* fish cell line RTL-W1) were also conducted with the leachates. No effects were observed in earthworms and plants at any tested concentration; reduction in the respiration rate (<5%) of soil microorganisms was detected. Earthworm/soil bioconcentration factors decreased with the increase in soil concentrations and were higher for the York soil. Effects on daphnids were observed in tested leachates; based on measured levels of ivermectin in the leachates an EC₅₀ of about 0.5 µg L⁻¹ can be estimated. Comparisons based on toxicity data and equilibrium partitioning confirmed that the main risk is expected to be related to the high sensitivity of cladocerans. The results confirm that MS·3 systems are cost-effective tools for assessing the impact of veterinary pharmaceuticals when applied to agricultural land, as previously demonstrated for antimicrobials.

Additional key words: effects; ivermectin; higher tier ecotoxicity test; soil microcosm; veterinary medicines.

Resumen

Valoración ambiental de medicamentos veterinarios en suelos agrícolas utilizando sistemas multi-especies (MS·3): la ivermectina como caso de estudio

La evaluación del riesgo ambiental de la ivermectina recibe atención. Este trabajo considera la capacidad de microcosmos terrestres (MS·3) para evaluar el impacto de medicamentos veterinarios, utilizando ivermectina como modelo. Se realizaron experimentos en MS·3 con dos suelos europeos, uno de una zona agrícola cercana a Madrid, España y otro de una explotación cerca de York, UK. Los suelos fueron fortificados con ivermectina considerando rangos de 0,01 a 10 mg kg⁻¹ con suelo de Madrid y de 0,1 a 100 mg kg⁻¹ con suelo de York. En el MS·3 de Madrid se evaluaron los efectos sobre lombrices, plantas y microorganismos. En los lixiviados, se ensayó la toxicidad en organismos acuáticos (algas, invertebrados y ensayos *in vitro*, con líneas celulares de peces RTL-W1). Las lombrices y las plantas no mostraron efecto a las concentraciones consideradas; sí se observó reducción en la tasa de respiración (<5%) de microorganismos. Los factores de bioconcentración en lombriz disminuían conforme aumentaba la concentración de ivermectina en suelo y éstos fueron mayores en lombrices expuestas al suelo de York. Hubo efectos sobre daphnidos expuestos a lixiviados; basado en los niveles de ivermectina medidos en lixiviados, se estimó una EC₅₀ de 0,5 µg L⁻¹. La comparación entre datos de toxicidad y métodos de equilibrio de partición confirma que el principal riesgo de la ivermectina debe esperarse en daphnias debido a la alta sensibilidad de cladóceros. Los resultados confirman que los MS·3 pueden considerarse una herramienta útil para la evaluación de impacto ambiental de medicamentos veterinarios en suelos agrícolas, como ya se demostró previamente con antimicrobianos.

Palabras clave adicionales: efectos; ivermectina; medicamentos veterinarios; microcosmos terrestre; prueba de ecotoxicidad de alto nivel.

* Corresponding author: carbonel@inia.es

Received: 13-10-10; Accepted: 06-04-11.

Introduction

Ivermectin, an avermectin parasiticide of high environmental concern, is broadly used as endo- and ecto-antiparasitic drug for treating livestock (Nessel *et al.*, 1989; Alvinerie *et al.*, 1998; Edwards *et al.*, 2001). Pharmacokinetic studies demonstrated a moderate metabolism, and significant amounts of the parent drug have been found in the dung of several livestock species (Alvinerie *et al.*, 1998; Fernández *et al.*, 2009). The main environmental concerns when treating animals with ivermectin are associated to their dung excretion, representing a potential risk to dung and soil fauna and also to aquatic organisms due to runoff losses from manure or slurry applications (Halley *et al.*, 1989; Van den Brink *et al.*, 2005). Aquatic organisms, in particular freshwater crustaceans such as amphipods and cladocerans, are the most sensitive organisms (Edwards *et al.*, 2001; Garric *et al.*, 2007). However, the concentrations measured in dung can also provoke toxic effects on terrestrial organisms (Edwards *et al.*, 2001; Jensen *et al.*, 2003; Floate *et al.*, 2005; Fernández *et al.*, 2009). During the last two decades, the environmental risk assessment of veterinary medicines including ivermectin has received significant attention (Halley *et al.*, 1989; Van den Brink *et al.*, 2005). The concerns were considered by regulatory bodies and the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products developed specific guidance (VICH, 2000, 2004), which have been implemented in the European Union (EMA, 2008). Specific provisions are considered for antiparasitic drugs, due to their high toxicity for certain taxonomic groups. Regulatory guidance focus on the use of standardised procedures and assays, which were originally developed for assessing other chemical groups; however, pharmaceuticals are designed for accomplish very precise biological functions, and are typical example for considering targeted environmental risk assessments (Tarazona *et al.*, 2010). Micro and mesocosms have been considered as a particularly useful method for targeting the environmental assessment of pharmaceuticals (Van den Brink *et al.*, 2005). Veterinary medicines are mostly released into the agro-sphere, either directly during pasture or when using manure as agricultural fertilizer. The multi-species soil system (MS-3) was developed by our group

as a simplified soil microcosms specifically designed for assessing effects on agricultural land (Fernández *et al.*, 2004; Boleas *et al.*, 2005a,b). The system balances the benefits of standardisation with the needs for realistic conditions, combining the toxicity endpoints measured in several OECD guidelines in a cost-effective assay reproducing realistic agricultural soil conditions. This higher tier ecotoxicity test has been designed as an artificially assembled soil microcosm where soil macro-organisms, plants and invertebrates, are added into a sieved column of natural soil that provides a complex microbial community. The capability of MS-3 soil microcosms for addressing the environmental risk of veterinary pharmaceuticals has been tested elsewhere (Montforts *et al.*, 2003), and several studies on antimicrobial drugs have been conducted (Boleas *et al.*, 2005a,b). This paper assess the capacity of the MS-3 soil microcosm as a cost-effective screening tool for targeting the environmental impact assessment of veterinary drugs. Ivermectin was selected as a case model as this drug combines a set of remarkable characteristics governing its biocidal activity and environmental concerns. Ivermectin has a high molecular weight, above the threshold which for general chemicals is assumed as indicator of low toxicity due to inability for crossing cellular membranes (ECB, 2003). It has a low solubility in water, a log K_{ow} above 3 and it sorbs strongly to soil, with a soil organic carbon distribution coefficient (K_{oc}) of 12,600-25,800 (Bloom and Matheson, 1993) although the presence of cations such as Ca^{2+} leads to decreasing soil sorption (Krogh *et al.*, 2008). Moreover, ivermectin has a high acute and chronic toxicity for several taxonomic groups (Halley *et al.*, 1989) with cladocerans being extremely sensitive (Garric *et al.*, 2007). Therefore, the MS-3 ivermectin results complement our previous observations for antimicrobial drugs.

Material and methods

Characteristics of the soils

MS-3 experiments were made up of two European loamy sand soils, from the Mediterranean and Atlantic eco-regions, covering a wide range of organic matter and nutrient content. The low organic matter Medi-

terranean soil was collected at a research facility located 40 km northeast of Madrid, Spain. The high organic matter Atlantic soil was collected in a farm area close to York, UK. Both soils were collected in sites not exposed to ivermectin for at least the last 10 years and, consequently, background concentrations of ivermectin were below the detection level. The Madrid soil has been also not treated with pesticides, fertilizers or soil amendments. This situation could not be confirmed for the York soil, and some leachate control samples provoked toxicity in daphnids, thus the York soil was only used for characterising the environmental fate of ivermectin. Soil samples were taken within the top 20 cm of the soil layer, sieved through a 2 mm mesh and homogenised before use. Table 1 shows the physico-chemical characteristics of both soils used in our MS·3 experiments. These two soils were classified as loamy sand soils, showing differences in the organic matter content (0.32% and 2.32% for Madrid and York soils, respectively) and the pH (8.30 for Madrid soil and 6.30 for York soil, respectively). These differences could play an important role in the behaviour (potential leaching, availability) of contaminants. The ivermectin

soil-water distribution coefficient (K_d) varied from 57 (Madrid soil) to 396 (York soil) L kg⁻¹ (Krogh *et al.*, 2008).

Multi-species-soil-system (MS·3) design

The MS·3 is an artificial assemblage of soil macro-organisms (*i.e.* plants, invertebrates) lying on homogeneous columns of sieved natural soil (for a full description see Fernández *et al.*, 2004) allowing combined assessments of fate and effects. Soil columns were assembled in PVC cylinders (20 cm internal diameter and 30 cm high), with a leachate collection device attached at the bottom of each column. The columns were maintained in a climate-control room with a 16 h light/8 h dark photoperiod (1,200 lux \pm 13% CV), temperature of 21 \pm 1°C and humidity of 55-60%.

The MS·3 assess simultaneously fate properties and the sensitivity of several taxonomic groups under realistic exposure conditions. This study was used as screening tool, using two largely different soils and distributing concentrations in orders of magnitude.

Table 1. Physico-chemical properties of Madrid and York soils used in the MS·3 studies

	Madrid soil	York soil
Moisture (%)	0.50	1.00
pH	8.50	6.30
EC (dS cm ⁻¹)	0.16	0.13
Organic matter (%)	0.32	2.65
N-NO ₃ ⁻ (mg kg ⁻¹)	< 1	15
P (mg kg ⁻¹)	19	49
K (mg kg ⁻¹)	107	56
Mg (mg kg ⁻¹)	94	169
CaCO ₃ equiv. (%)	< 4	< 4
Ca (mg kg ⁻¹)	3,795	1,191
Na (mg kg ⁻¹)	37	46
Sand (0.05 < D < 2 mm) (%)	79	68.80
Silt (0.02 < D < 0.05) (%)	8.80	6
Silt (0.002 < D < 0.02) (%)	6.80	13.30
Clay < 0.002 mm (%)	5.40	11.90
Soil type (USDA)	Loamy sand	Loamy sand
N-NH ₃ (mg kg ⁻¹)	4.70	8
CEC (meq 100 g ⁻¹)	4.70	10
Cu (mg kg ⁻¹)	6	27
Fe (mg kg ⁻¹)	32	852
Zn (mg kg ⁻¹)	21	58
N Kjeldahl (%)	0.04	0.16
WHC _{max} (%)	21.33	21.93

D: diameter size. CEC: cation exchange capacity. WHC_{max}: maximum water holding capacity.

Test compound and soil fortification

Ivermectin (CAS N°. 70288-86-7, purity 94% B_{1a} and 2.8% B_{1b}) was purchased from Sigma Aldrich. For this screening assessment, ivermectin concentrations were assayed in orders of magnitude, ranging from 0.01 to 10 mg iv kg⁻¹ dry wt for (MS·3-A) and 0.1 to 100 mg iv kg⁻¹ dry weight for (MS·3-B). Spring water was added to all soils until reaching 40% of the maximum water holding capacity (WHC_{max}). All MS·3, controls and iv-fortified, were assayed in triplicates.

Soil invertebrates and plants

The selected taxonomic groups used in the MS·3 experiments were terrestrial invertebrates (*Eisenia fetida*, Savigny) and mono and dicotyledonous plants. *Eisenia fetida* has been cultured for several generations in our laboratory and certified seeds of the vascular plants wheat *Triticum aestivum* L, rape *Brassica napus* L, red clover *Trifolium pratense* L and common vetch *Vicia sativa* L, were kindly supplied by the Spanish Office of Plant Varieties. These species are recommended in OECD Guidelines 207 and 208 (OECD, 1984a,b).

MS-3: experimental protocol

MS-3 systems were saturated with spring water (750-1,000 mL in each MS-3 column) and left overnight. Then, groups of 10 *E. fetida* adults, 10 *T. aestivum* seeds, and 10 *T. pratense* seeds for (MS-3-A), or 10 *V. sativa* seeds for (MS-3-B), were introduced in each column. One hour later, columns were irrigated with 100 mL of spring water. A fixed volume of 100 mL of spring water was added (from Monday to Friday) and leachates were collected at days 0, 14 and 21.

Electrical conductivity and pH were measured in leachates samples at the beginning and on days 7, 14 and 21, using a pH-meter, (Orion 520A, Boston, MA, USA) and a conductivimeter (Crison, Barcelona, Spain).

Ivermectin analysis

Sample extraction and clean-up procedures were based on the methodology described by Kolar *et al.* (2004), for the determination of abamectine and doramectine in sheep faeces. HPLC analysis followed the procedure described by Sun *et al.* (2005). Briefly, the procedures were as described below.

Ivermectin soil sample extraction and clean-up procedure

In a 100 mL centrifuge tube, 20g of soil sample were mixed with 10 g of anhydrous sodium sulphate; after the addition of 40 mL of acetonitrile containing 50 μ L of triethylamine (Sigma-Aldrich T0886), the mixture was extracted in an ultrasonic bath for 15 min; the acetonitrile phase was separated by centrifugation (5 min, 3,500 rpm). A volume of 30 mL of the acetonitrile phase was mixed with 70 mL of MilliQ water before SPE using HLB cartridges (Strata-X 33 μ m 60 mg/3 mL, Phenomenex 8B-S100-UBJ), previously activated with: 5 mL acetonitrile, 5 mL acetonitrile/MilliQ water 30:70 (v:v) and 5 mL water MilliQ. After washing (5 mL acetonitrile/MilliQ water 30/70) and drying, iv was eluted from the SPE cartridge with 5 mL of acetonitrile. Then, 0.2 mL of internal standard acetonitrile solution (200 ng mL⁻¹ abamectin 46392 PESTANAL, Riedel-de-Häen) were added to eluate and then evaporated to dry by vacuum using a Genevac EZ-2 evaporation system at its maximum temperature (30°C). After drying, the residue was derivatized with 1-methylimi-

dazol following the procedure according to Sun *et al.* (2005).

Ivermectin leachate sample extraction and clean-up procedure

A volume of 50 mL leachate was mixed with 50 μ L triethylamine and 20 mL acetonitrile. Thereafter, SPE were performed using HLB cartridges (Strata-X 33 μ m 60 mg/3 mL, Phenomenex 8B-S100-UBJ) with the same procedure used for soil samples.

Ivermectin earthworm sample extraction and clean-up procedure

In a 50 mL glass vessel, a pool of 10 earthworms (from each MS-3 column) were weighed. Acetonitrile (50 mL) and 50 μ L of triethylamine were added before homogenization (IKA T25 digital ULTRA-TURRAX). After washing with another 20 mL of acetonitrile, the slurry was centrifuged (3,500 rpm, 5 min). Acetonitrile extract (30 mL) were filtered (Sartorius glasfibre filter) and diluted with 70 mL of MilliQ water. Then SPE was performed using HLB cartridges (Strata-X33 μ m 60 mg: 3 mL, Phenomenex 8B-S100-UBJ) with the same procedure used in soil samples.

Validation and calibration curves

Methods for soil and leachate were validated using iv-fortified (soil and leachate) control samples. Calibration curves were prepared daily using fortified samples, prepared by adding iv acetonitrile solution in order to obtain standard samples containing iv concentrations ranging from 0.0025 to 0.05 ng mL⁻¹ in leachates samples and from 0.0625 to 2.5 ng g⁻¹ wet weight in soil samples. Standard samples were extracted in parallel with unknown samples. The method for earthworms was not validated.

Ivermectin HPLC analysis

A volume of 100 μ L of standard or sample solution was injected in a HPLC system equipped with Waters 2695 Separations Module, Waters 2475 Fluorescence detector and Millennium 32 chromatographic software. The chromatographic conditions used were the follow-

ing: Column Luna 5 μm C18 (2), 250×4.60 mm (Phenomenex) at 40°C , mobile phase: MilliQ water: acetonitrile 3:97 at 1.5 mL min^{-1} and fluorescence detection at 365 nm (Ex) and 475 nm (Em). Quantitation was performed by internal standard procedure.

Recoveries, limits of detection and quantization

The percentage of recovery was determined on 4 samples (50 mL) of control leachate fortified with $2\text{ }\mu\text{g L}^{-1}$ ($96\% \pm 2\%$) and on 3 samples (15 g) of control soil spiked at $10\text{ }\mu\text{g kg}^{-1}$ ($91.5\% \pm 2.09\%$) and $50\text{ }\mu\text{g kg}^{-1}$ ($85\% \pm 3\%$). Following the above described sample preparations, detection limits were 0.02 ng mL^{-1} and 0.10 ng g^{-1} for leachate and soil samples, respectively. Quantifications limits were established at the lowest calibration point and detection limit were established at 2.5 times below the quantization limit, with a signal/noise ratio higher than 10.

Ecotoxicological effects assessment

Effects on plants (seed germination) and earthworms (mortality) were determined in the Madrid soil at the end of the 21 exposure days.

Soil microbial function effects were determined by measuring soil enzymatic dehydrogenase and phosphatase (Carbonell *et al.*, 2000) activities, and substrate induced respiration rate (Fernández *et al.*, 2004). Measurements were conducted at three depths of the soil column (top, medium and bottom). Respiration rate was analyzed according to the procedure described by Fernández *et al.* (2004). The effect of iv on soil microbial respiration, measured as glucose-induced CO_2 production rate, was determined at 22°C using BacTrac 4300 system (SY-LAB, GmbH, P.O. Box 47, A-3002 Purkersdorf, Austria); the methodology is based on the variation of conductivity of a 0.2% KOH water solution. Respiration rates were calculated in the linear phase of the respiration curves.

Using leachates, indirect effects on aquatic organisms were assessed. *Daphnia* acute immobilisation test (OECD guideline 202; OECD, 2004), alga growth inhibition test (OECD guideline 201; OECD, 2002) and *in vitro* toxicity test on fish cell line RTL-W1 (Babin and Tarazona, 2005) were performed.

The *Daphnia magna* immobilisation test was conducted for each leachate in duplicate, in a thermos-

tatised chamber ($20 \pm 1^\circ\text{C}$) with 16 h light/8 h dark photoperiod. Leachates from untreated MS-3 were carried out in parallel as controls; 10 daphnids (<24 h-old) were exposed directly in leachate (15 mL) and 48 h later, daphnids unable to swim for 15 s after gentle stirring were considered as immobile. The EC_{50} 48 h was calculated ($\mu\text{g L}^{-1}$) based on the measured concentration of iv in the leachates.

The unicellular green algae (*Chlorella vulgaris*) growth inhibition tests were conducted following the adaptation of the freshwater algae and cyanobacteria, growth inhibition test, guideline 201 (OECD, 2002), using 96-well microplates (Ramos *et al.*, 1996). This method evaluates the algal growth using 96-well microplates by absorbance (450 nm) and/or fluorescence (430 Ex/680 Em) multiwell plate reader (TECAN-Genius spectrofluorometer); when interference of effluent samples with absorbance and/or fluorescence determination was detected, microscopic counts on aliquots of each treatment were performed. The use of alternative *in vitro* methods represents a possibility for assessing the effects of chemicals (Worth and Balls, 2002). *In vitro* toxicity tests on fish cell line RTL-W1 were performed with leachates; EROD activity, β -galactosidase activity and cellular viability (neutral red assay) were analyzed following the procedures described in Babín and Tarazona (2005), with leachates reconstituted with 10x EMEM media. The use of a concentrated media diluted directly with the sample to be tested allowed the exposure of cell cultures to concentrations around 75% of the original sample. Water controls (water reconstituted 10x EMEM media) were run in parallel. In addition, total protein content of the cells was measured using the Kenacid blue protein (KBP) assay (Knox *et al.*, 1986). The presence of organic toxic chemicals was indicated by the induction or inhibition of the cytochrome CYP1A activity measured as 7-ethoxyresorufin-O-deethylase (EROD) activity (Babin and Tarazona, 2005), cellular defense was predicted by a β -galactosidase (β -GAL) assay (Babín and Tarazona, 2005). To quantify fluorescence (kinetic way) and absorbance endpoints, a TECAN-Genius spectrofluorometer was used.

Statistical analysis

Statistical differences associated to the iv treatment were analysed by one-way ANOVA test and least signi-

ficant differences multiple range test. EC50 values were estimated by probit analysis. All statistic estimations were conducted using the Statgraphics Plus Ver. 5.1 software.

Results

Chemical properties of leachates

Volume, pH and electrical conductivity of collected leachates are summarized in Table 2. The pH values were kept constant during the exposure time and no significant differences between control and treatments were observed. In general, leachates from (MS·3-B) showed higher EC values than leachates from (MS·3-A).

Ivermectin analysis in MS·3 soil and leachates

The initial iv concentrations measured in fortified soils were: 0.008 ± 0.003 ; 0.079 ± 0.020 ; 0.630 ± 0.319 and 5.71 ± 4.47 mg kg⁻¹ dry weight for (MS·3-A) and 0.204 ± 0.004 ; 1.198 ± 0.017 ; 12.965 ± 0.088 and 106.695 ± 5.542 mg kg⁻¹ dry weight for (MS·3-B). Table 2 shows the iv concentration in the collected leachates at different times. Ivermectin was not detected (LD = 0.020 ng L⁻¹) in leachates from the lowest concentration (0.01 mg kg⁻¹ dry weight); but most other samples showed detectable concentrations. Concentrations were mostly related to the soil nominal concentration; and in general, leachates from Madrid soil tended to be have higher concentrations than leachates from York soil; however, very high concentrations were

Table 2. Chemical analysis of leachates from Madrid (MSD·3-A) and York (MS·3-B) leachates

Madrid leachates	Date (d)	Ivermectin concentration in (MS·3-A) leachates: mean \pm standard deviation (Variation coefficient)				
		Control	0.01 mg kg ⁻¹ soil	0.1 mg kg ⁻¹ soil	1 mg kg ⁻¹ soil	10 mg kg ⁻¹ soil
Volume (mL)	(0,7,14,21)	137.42 \pm 63.06 (45.89)	125.38 \pm 60.69 (48.40)	103.08 \pm 62.69 (48.40)	86.50 \pm 62.68 (72.46)	52.58 \pm 60.74 (115.52)
pH	(0,7,14,21)	8.26 \pm 0.22 (2.64)	8.36 \pm 0.18 (2.19)	8.36 \pm 0.17 (2.01)	8.36 \pm 0.20 (2.42)	8.28 \pm 0.21 (2.48)
EC (μ S cm ⁻¹)	(0,7,14,21)	1,109 \pm 103.20 (9.31)	1,110 \pm 90.51 (8.22)	1,074 \pm 91.81 (8.55)	1,200 \pm 168.07 (13.99)	1,216 \pm 277.59 (22.83)
Iv (ng mL ⁻¹)	0	ND	ND	ND	2.7*	23.3**
	7	ND	ND	1.2**	2.2**	46.1*
	14	ND	ND	ND	1.2 \pm 0.3	20.4*
York leachates	Date (d)	Ivermectin concentration in (MS·3-B) leachates: mean \pm standard deviation (Variation coefficient)				
		Control	0.1 mg kg ⁻¹ soil	1 mg kg ⁻¹ soil	10 mg kg ⁻¹ soil	100 mg kg ⁻¹ soil
Volume (mL)	(0,7,14,21)	177.17 \pm 89.89 (50.70)	190.08 \pm 115.83 (60.94)	201.33 \pm 115.72 (57.48)	163.08 \pm 103.17 (63.26)	133 \pm 75.84 (57.02)
pH	(0,7,14,21)	7.16 \pm 0.33 (4.56)	7.63 \pm 0.70 (9.19)	7.25 \pm 0.66 (9.12)	7.18 \pm 0.50 (7)	6.93 \pm 0.22 (3.11)
EC (μ S cm ⁻¹)	(0,7,14,21)	1,496 \pm 250 (16.69)	1,456 \pm 350 (24.02)	1,497 \pm 400 (26.70)	1,490 \pm 349 (24.77)	1,184 \pm 269 (22.72)
Iv (ng mL ⁻¹)	0	ND	0.02 \pm 0.04	ND	0.195 \pm 0.190	ND
	7	ND	ND	ND	1.04 \pm 0.41	3.15 \pm 1.76
	14	ND	4.21 \pm 0.37	14.07 \pm 24.17	4.96 \pm 3.13	8.47 \pm 7.69
	21	ND	0.27 \pm 0.36	0.30 \pm 0.33	0.28 \pm 0.49	1.84 \pm 2.97

ND < LD = 0.02 ng mL⁻¹. *: One value. **: Mean of two values.

Table 3. Endpoints and results of a multi-species soil system (MS-3) assay with ivermectin

Endpoint	Time (d)		Control	Dose (mg Iv kg ⁻¹ soil)			
				0.01	0.1	1	10
<i>E. fetida</i> mortality (%)	21		0	0	0	0	0
Seed germination (%)	21	<i>T. aestivum</i>	93	97	93	90	93
		<i>V. sativa</i>	93	97	100	90	97
Soil dehydrogenase (%)	0			NS	NS	NS	NS
	21	Top		NS	NS	NS	42.02 ± 6.55 ^a (-34.63)
		Medium		NS	NS	NS	NS
		Bottom		NS	NS	NS	NS
Soil phosphatase (%)	0			NS	NS	NS	NS
	21	Top		NS	NS	NS	37.61 ± 2.53 ^a (-9.47%)
		Medium		22.09 ± 2.21 ^a (-20.17%)	NS	NS	22.33 ± 2.28 ^a (-19.42%)
		Bottom		NS	NS	NS	39.46 ± 4.75 ^a (-11.27%)
Respiration rate (%)	0			NS	1.10 ± 0.04 ^a (-2.30%)	1.07 ± 0.04 ^a (-4.96%)	1.07 ± 0.05 ^a (-5.01%)
	21	Top		NS	NS	NS	NS
		Medium		NS	NS	NS	NS
		Bottom		NS	NS	NS	NS

^a Statistically significant differences for the iv soil concentration in the one-way ANOVA test ($p < 0.05$). NS: not significant. Number in parenthesis correspond to % inhibition (-) versus control.

observed in the leachates from the York soil treated with 0.1 and 1 mg kg⁻¹. A similar pattern was observed for both soils, with leachate concentrations peaking at day 14 for the York experiment in all treatments and at different days in different treatments for the Madrid study.

Ivermectin effects on terrestrial invertebrates, plants and microorganisms in MS-3

The effect on earthworms, plants, and microorganisms observed in the Madrid soil are shown in Table 3. Only

soil microbial functions were affected, mostly at the highest iv concentration tested. Slightly reductions in the respiration rate (< 5%) were observed at the beginning of the exposure but were not longer observed at day 21.

Ivermectin concentrations in *Eisenia fetida* after 21 exposure days are shown in Figure 1.

A direct relationship between soil and earthworm concentrations was observed; however, a non-linear inverse relationship was observed for the earthworm-soil bioaccumulation factors, which are presented in Figure 2. It should be noticed that the earthworm/soil

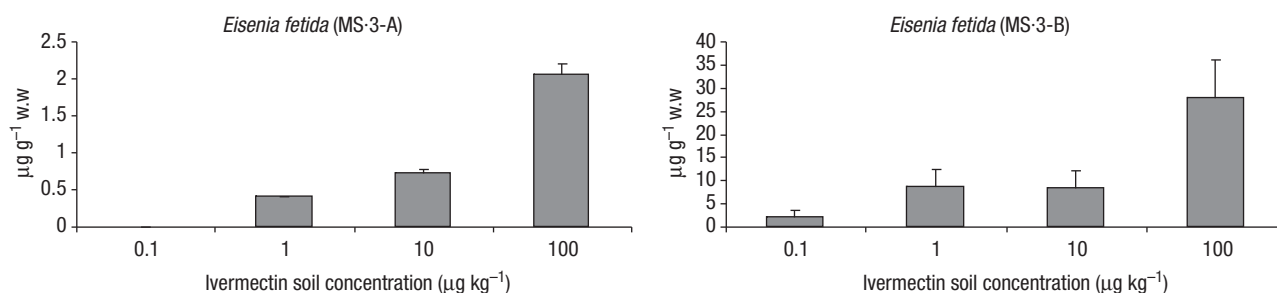


Figure 1. Ivermectin concentration in *Eisenia fetida* collected after exposure in MS-3 experiments from Madrid (MS-3-A) and York (MS-3-B) soils.

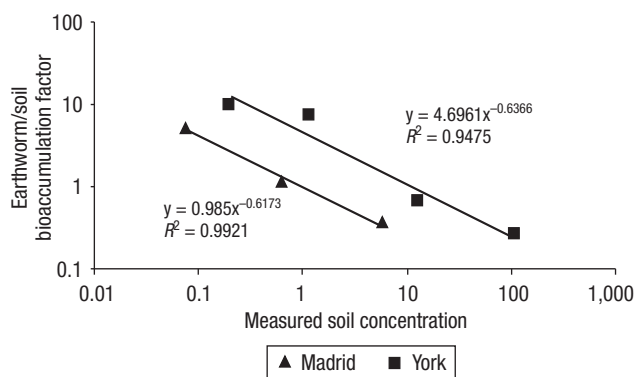


Figure 2. Relationship between ivermectin soil concentration and earthworm/soil bioconcentration factors in *Eisenia fetida* from Madrid (MS-3-A) and York (MS-3-B) soils.

bioaccumulation factors were higher for the York than for the Madrid soil.

Ivermectin effects on aquatic organisms

Effects on *Daphnia magna* immobilisation was observed in the leachates. Figure 3 shows the concentration/response relationship based on measured levels of iv in the leachates; an EC_{50} about $0.5 \mu\text{g L}^{-1}$ was estimated. Concentrations at and above $1 \mu\text{g L}^{-1}$ resulted in a 100% immobilisation. It should be noted that this concentration was exceeded at least at one time point in the leachates from all soils treated with iv concentrations of 0.1 mg kg^{-1} or higher. On the other hand, algae and the RTL-W1 fish cell line did not seem to be affected by iv at the concentrations assayed.

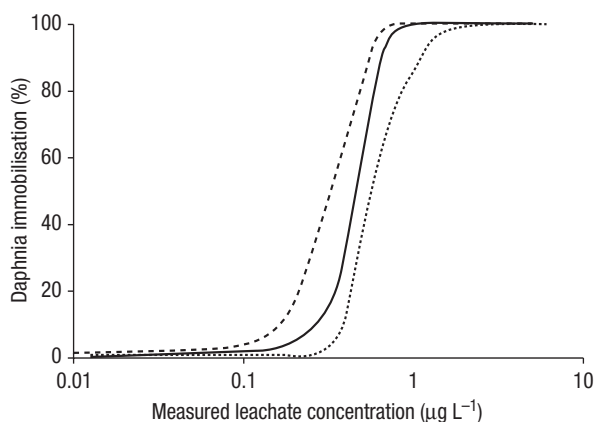


Figure 3. Concentration response relationship between the measured ivermectin concentration in the Madrid soil leachates and the observed immobilisation in *Daphnia magna* (fitting adjustment and 95% confidence intervals).

Daphnia magna immobilisation was the most sensitive endpoint.

Discussion

The early identification of the most relevant receptors in an environmental risk assessment may avoid unnecessary testing focusing the resources on those receptors representing the highest level of risk. Within the frame of the ERApharm project several ways for targeting the environmental risk of pharmaceuticals have been proposed (Tarazona *et al.*, 2010). One possibility is described as the risk line approach. The risk line represents a further development of the exposure scenario concept; each line connects each environmental release with each relevant ecological receptor, sorting out all the processes associated to the exposure of that particular receptor. Screening tools can be used to identify the relevance of each risk line, prioritizing further refinements if needed. The default conditions established for setting generic scenarios and equilibrium partitioning methods adapted to the characteristics of the substance can be used for preliminary comparisons among organisms exposed through different media (*e.g.* water, sediment or soil). The MS-3 microcosm also offers some comparative assessments by measuring simultaneously the toxicity of a soil and their leachates obtained under realistic conditions to soil organisms and aquatic species respectively.

The toxicity of iv to aquatic organisms is characterized by a huge divergence in sensitivity among species. Cladocerans are one-to-two, three and five orders of magnitude more sensitive than other crustaceans, fish and mollusks, respectively (Halley *et al.*, 1989; Davies *et al.*, 1997; Garric *et al.*, 2007). However, this diversity is not observed for soil dwelling invertebrates. Collembolans are just one order of magnitude more sensitive than earthworms and enchytraeids (Halley *et al.*, 1989; Gunn and Sadd, 1994; Römbke *et al.*, 2010). The acute to chronic ratio is also much higher for cladocerans (four orders of magnitude according to Garric *et al.* (2007) than for soil invertebrates such as collembolan and earthworms (Halley *et al.*, 1989; Gunn and Sadd, 1994; Jensen *et al.*, 2003; Römbke *et al.*, 2010). The equilibrium partitioning method is used for extrapolating aquatic toxicity data to the soil compartment (Bockting *et al.*, 1993). Using the iv K_{oc} range and the default values recommended in the EU guidelines (ECB, 2003; ECHA, 2008), the acute and chronic

toxicity data available for aquatic species (Halley *et al.*, 1989; Davies *et al.*, 1997; Garric *et al.*, 2007) can be compared with those observed for soil dwelling organisms (Halley *et al.*, 1989; Gunn and Sadd, 1994; Jensen *et al.*, 2003; Römbke *et al.*, 2010). Based on the data published by Garric *et al.* (2007), cladocerans would be about three and six orders of magnitude more sensitive than soil organisms for acute and chronic effects, respectively. However, other crustaceans and fish would be just slightly more sensitive than soil invertebrates. The screening MS-3 is consistent with this assessment, and offers the same information with a single cost-effective test. The additional advantage is that the comparison is not based on soil pore water equilibrium partitioning but on direct toxicity testing of soil leachates obtained under realistic environmental conditions. The leachates are toxic to cladocerans at concentrations at least two orders of magnitude below those toxic for terrestrial plants and earthworms.

The comparison of the EC_{50} for *Daphnia magna* observed in the leachates tests (about $0.5 \mu\text{g L}^{-1}$) with the reported values in standardized test (5.7 to 25 ng L^{-1}) by Garric *et al.* (2007), suggests that only about 1-5% of the iv present in the leachate would be bioavailable. The formation of colloids and complexes with inorganic matter may explain this low bioavailability (Krogh *et al.*, 2008). It should be noted that when the leachate concentrations are corrected by the bioavailability factor calculated for *Daphnia magna*, the resulting concentrations are lower than those expected to be toxic for fish, and therefore would explain the lack of toxicity observed for the RT1-W1 fish cell line. The lack of effects on algae can be also explained by the low sensitivity of this taxonomic group to algae (Halley *et al.*, 1989). A soil sorption mechanism different from lipophilicity would also explain the higher concentrations observed in the York *versus* the Madrid leachates at concentrations of 1 mg kg^{-1} soil or lower, despite the much higher organic matter content of the York soil. The contribution of calcium as a factor enhancing iv mobility in soil, hypothesized by the same authors, would suggest higher concentrations in the Madrid leachates, but this was only observed at concentrations of 10 mg kg^{-1} and higher. Concentration related tendencies were also observed for earthworm bioaccumulation. A higher bioconcentration was observed for the York soil at all tested concentrations. The comparison of the measured earthworm concentrations with the NOEL of $0.1 \text{ mg kg}^{-1} \text{ bw}^{-1}$ for maternal-toxicity effects in a mouse teratogenicity study selected by

the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and EMEA 2004, suggest concern for secondary poisoning of terrestrial wildlife even at the lowest soil concentrations. These results confirm with real data the recent assumptions from De Lange *et al.* (2009) based on expert judgment and multicriteria analysis. The screening MS-3 soil microcosm would target the environmental impact assessment of ivermectin to the following risk-lines (for details, see Tarazona *et al.*, 2010): 1) Release to agricultural soil \rightarrow runoff/leaching/drainage to aquatic bodies \rightarrow risk to aquatic invertebrates. 2) Release to agricultural soil \rightarrow accumulation into soil dwelling invertebrates \rightarrow risk of secondary poisoning to terrestrial wildlife.

The assessment of the extensive amount of information available for iv demonstrates that in fact these are the most relevant risk (Liebig *et al.*, 2010), confirming the capacity of the MS-3 microcosms as screening tool. It should be noted that the potential risk for dung fauna is not covered in this assessment. The main limitations of the MS-3 system to be used for pharmaceuticals and other chemicals with specific mechanisms of action, is the use of acute instead of chronic endpoints. Although our group is trying to incorporate chronic endpoints into the system, a reproducible and cost-effective assemblage has not been obtained yet. These limitations can be solved through the combination of the MS-3 with reproduction assays on daphnids and collembolans. It should be noted this test battery should still be highly cost-effective for targeting the environmental assessment of pharmaceuticals and other chemicals. The results of the MS-3 presented here, combined with the single species laboratory studies conducted by other ERA-Pharm partners on daphnids (Halley *et al.*, 1989) and collembolans (Römbke *et al.*, 2010) offer sufficient information for a targeted assessment of this antiparasitic drug, as alternative to a full environmental assessment also provided by the project (Liebig *et al.*, 2010). These results are in line with those previously published for antimicrobials, confirming that MS-3 systems are cost-effective tools for assessing the impact assessment of veterinary pharmaceuticals in agricultural land.

Acknowledgments

This work was funded by the European Commission under the sixth framework program in the STREP

«Environmental risk assessment of pharmaceuticals» (ERAPharm, SSPI-CT-2003-511135).

References

- ALVINERIE M., SUTRA J.F., GALTIER P., LIFSCHITZ A., VIRKEL G., SALLOVITZ J., LANUSSE C., 1998. Persistence of ivermectin on plasma and faeces following administration of a sustained-release bolus to cattle. *Res Vet Sci* 66, 57-61.
- BABÍN M.M., TARAZONA J.V., 2005. *In vitro* toxicity of selected pesticides on RTG-2 and RTL-W1 fish cell lines. *Environ Pollut* 135, 267-274.
- BLOOM R.A., MATHESON III J.C., 1993. Environmental assessment of avermectins by the US Food and Drug Administration. *Vet Parasitol* 48, 281-294.
- BOCKTING G.M., VAN DE PLASSCHE E.J., STRIJS J., CANTON J.H., 1993. Soil-water coefficients for organic compounds. RIVM. Bilthoven. RIVM /679110013.
- BOLEAS S., ALONSO C., PRO J., FERNÁNDEZ C., CARBONELL G., TARAZONA J.V., 2005a. Toxicity of the antimicrobial oxytetracycline to soil organisms in a multi-species-soil system (MS-3) and influence of manure co-addition. *J Hazard Mater* 122, 233-241.
- BOLEAS S., ALONSO C., PRO J., BABÍN M.M., FERNÁNDEZ C., CARBONELL G., TARAZONA J.V., 2005b. Effects of sulfachlorpyridazine in MS-3-arable land: a multi-species soil system for assessing the environmental fate and effects of veterinary medicines. *Environ Toxicol Chem* 24, 811-819.
- CARBONELL G., PABLOS M.V., GARCÍA P., RAMOS C., SÁNCHEZ P., FERNÁNDEZ C., TARAZONA J.V., 2000. Rapid and cost-effective multiparameter toxicity tests for soil microorganisms. *Sci Total Environ* 247, 143-150.
- DAVIES I.M., MCHENERY J.G., RAE G.H., 1997. Environmental risk assessment from dissolved ivermectin to marine organisms. *Aquaculture* 158, 263-275.
- DE LANGE H.J., LAHR J., VAN DER POL J.J., WESSELS Y., FABER J.H., 2009. Ecological vulnerability in wildlife: an expert judgment and multicriteria analysis tool using ecological traits to assess relative impact of pollutants. *Environ Toxicol Chem* 28, 2233-40.
- ECB, 2003. Technical guidance document (TGD) on risk assessment in support of Commission Directive 93/67/EEC on risk assessment of new notified substances, Commission Regulation (EC) No 1488/94 on risk assessment for existing substances, Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. Part II. Ispra (IT): European Commission Joint Research Centre. EUR 20418 EN/2.
- ECHA, 2008. Guidance on information requirements and chemical safety assessment. Chapter R.10: Characterization of dose (concentration)-response for environment, Helsinki. 65 pp.
- EDWARDS C.L., ATIYEH R.M., RÖMBKE J., 2001. Environmental impact of avermectins. *Rev Environ Contam T* 171, 111-137. ISSN 0179-553. Springer-Verlag, Germany.
- EMA, 2004. EMA/MRL/915/04-FINAL November 2004 Committee for medicinal products for veterinary use ivermectin (modification of maximum residue limits). Summary Report 5, London. 7 pp.
- EMA, 2008. Environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 and GL38. Doc. Ref. EMA/CVMP/VICH/790/03 /Final. London, Jan 2005.
- FERNÁNDEZ C., ALONSO C., BABIN M.M., PRO J., CARBONELL G., TARAZONA J.V., 2004. Ecotoxicological assessment of doxycycline in aged pig manure using multispecies soil systems (MS-3). *Sci Total Environ* 323, 63-69.
- FERNÁNDEZ C., SAN ANDRÉS M., PORCEL M.A., RODRÍGUEZ C., ALONSO A., TARAZONA J.V., 2009. Pharmacokinetic profile of ivermectin in cattle dung excretion, and its associated environmental hazard. *Soil Sediment Contam* 18, 564-575.
- FLOATE K.D., WARDHAUGH K.G., BOXALL A.B.A., SHERRATT T.N., 2005. Fecal residues of veterinary parasiticides: nontarget effects in the pasture environment. *Annu Rev Entomol* 50, 153-179.
- GARRIC J., VOLLAT B., DUIS K., PÉRY A., JUNKER T., RAMIL M., FINK G., TERNES T.A., 2007. Effects of the parasiticide ivermectin on the cladoceran *Daphnia magna* and the green alga *Pseudokirchneriella subcapitata*. *Chemosphere* 69, 903-910.
- GUNN A., SADD J.W., 1994. The effect of ivermectin on the survival, behaviour and cocoon production of the earthworm *Eisenia fetida*. *Pedobiologia* 38, 327-333.
- HALLEY B.A., JACOB T.A., LU AY.H., 1989. The environmental impact of the use of ivermectin: environmental effects and fate. *Chemosphere* 18, 1543-1563.
- JENSEN J., KROGH P.H., SVERDRUP L.E., 2003. Effects of the antibacterial agents tiamulin, olanquinox, and metronidazole and the antihelminthic ivermectin on the soil invertebrate species *Folsomia fimetaria* (Collembolla) and *Enchytraeus crypticus* (Enchytraeidae). *Chemosphere* 50, 437-443.
- KNOX P., UPHILL P.F., FRY J.R., BEUFORD J., BALLS M., 1986. The FRAME multicentre project on *in vitro* cytotoxicology. *Food Chem Tox* 24, 457-463.
- KOLAR L., KUZNER J., ERZEN N.K., 2004. Determination of abamectin and doramectin in sheep faeces using HPLC with fluorescence detection. *Biomed Chromatogr* 18, 117-124.
- KROGH K.A., SØEBORG T., BRODIN B., SØRENSEN B.H., 2008. Sorption and mobility of ivermectin in different soils. *J Environ Qual* 37, 2202-2211.
- LIEBIG M., ALONSO A.A., BLÜBAUM-GRONAU E., BOXALL A., BRINKE M., CARBONELL G., EGELER P., FENNER K., FERNÁNDEZ C., FINK G. *et al.*, 2010. Environmental risk assessment of ivermectin: a case study. *Integr Environ Assess Manag* 6, 567-587.

- MONTFORTS H.M.M., BACKWELL P., BOXALL A.B.A., HALLING-SØRENSEN B., HERMENSEN S., INGERSLEV F., JACOBSEN M., KAY P., LAAK T., MOLTSMANN J. *et al.*, 2003. Environmental risk assessment for veterinary medicinal products. Part 5. A guide to risk assessment of veterinary medicinal products used in animal husbandry. RIVM Report 601450018/2003. Bilthoven. pp. 78.
- NESSEL R.J., WALLACE D.H., WEHNER T.A., 1989. Environmental fate of ivermectin in a cattle feedlot. *Chemosphere* 18, 1531-1541.
- OECD, 1984a. Earthworm acute toxicity test. Guideline for testing of chemicals 207. Organisation for Economic Co-Operation and Development, Paris, France.
- OECD, 1984b. Terrestrial plants test. Guideline for testing of chemicals 208. Organisation for Economic Co-Operation and Development, Paris, France.
- OECD, 2002. Freshwater algae and cyanobacteria, growth inhibition test. Guideline for the testing of chemicals, draft revised guideline 201. Organisation for Economic Co-Operation and Development, Paris, France.
- OECD, 2004. *Daphnia* sp., acute immobilisation test. Guideline for testing of chemicals 202. Organisation for Economic Co-Operation and Development, Paris, France.
- RAMOS C., DE LA TORRE A., TARAZONA J.V., MUÑOZ M.J., 1996. Desarrollo de un ensayo de inhibición de *Chlorella vulgaris*, utilizando un test en microplacas. *Rev Toxicol* 13, 97-100. [In Spanish].
- RÖMBKE J., KROGH K.A., MOSER T., SCHEFFCZYK A., LIEBIG M., 2010. Effects of the veterinary pharmaceutical ivermectin on soil invertebrates in laboratory tests. *Arch Environ Contam Toxicol* 58, 332-40.
- SUN Y.J., DIAO X.P., ZHANG Q.D., SHEN J.Z., 2005. Bioaccumulation and elimination of avermectin B-1a in the earthworms (*Eisenia fetida*). *Chemosphere* 60, 699-704.
- TARAZONA J.V., ESCHER B., GILTROW E., SUMPTER J.P., KNACKER T., 2010. Targeting the environmental risk assessment of pharmaceuticals: facts and fantasies. *Integr Environ Assess Manag* 6, 603-613.
- VAN DEN BRINK P.J., TARAZONA J.V., SOLOMON K.R., KNACKER T., VAN DEN BRINK N.W., BROCK T.C.M., HOOGLAND J., 2005. The use of terrestrial and aquatic microcosm and mesocosms for the ecological risk assessment of veterinary pharmaceuticals. *Environ Toxicol Chem* 24, 820-829.
- VICH, 2000. International cooperation on harmonisation of technical requirements for registration of veterinary medicinal products. Environmental impact assessment (EIAs) for veterinary medicinal products (VMPs) – Phase I. VICH GL 6 (Ecotoxicity Phase I).
- VICH, 2004. International cooperation on harmonisation of technical requirements for registration of veterinary medicinal products. environmental impact assessment for veterinary medicinal products Phase II Guidance. VICH GL 38 (Ecotoxicity Phase II).
- WORTH A.P., BALLS M. (eds), 2002. Alternative (non-animal) methods for chemicals testing: current status and future prospects. A report prepared by ECVAM and the ECVAM Working Group on Chemicals. *ATLA* 30(Suppl. 1), 1-125.